

Placental growth factor induces *FosB* and *c-Fos* gene expression via Flt-1 receptors

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Abstract Placental growth factor (PIGF) is a member of the vascular endothelial growth factor (VEGF) family that binds specifically to Flt-1. The biological roles of PIGF and Flt-1 have not yet been defined and the signalling mechanisms mediating cellular actions of PIGF remain poorly understood. In human umbilical vein endothelial cells, VEGF and PIGF induced expression of both full-length *FosB* mRNA and an alternatively spliced variant, Δ *FosB*, with similar efficacy and kinetics. In contrast, PIGF induced *c-Fos* expression less strongly than VEGF, and whereas VEGF strongly upregulated tissue factor mRNA, PIGF had a negligible effect. PIGF induced *c-Fos* expression in porcine aortic endothelial cells specifically expressing Flt-1, and *FosB* expression in the monocytic RAW 264.7 cell line expressing endogenous Flt-1. These findings show for the first time that VEGF and PIGF induce mRNA expression of the transcription factors *FosB* and *c-Fos*, and suggest that these factors may play a role in the biological responses mediated by PIGF and Flt-1.

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1. Introduction

Vascular endothelial growth factor (VEGF or VEGF-A) is essential for endothelial cell differentiation and angiogenesis during development of the embryonic vasculature and plays a major role in neovascularisation in a variety of disease states [1,2]. Two protein tyrosine kinase receptors for VEGF, VEGFR2/KDR/Flk-1 and VEGFR1/Flt-1, are essential for embryonic vascular development [3,4], but signal transduction and biological responses triggered by VEGF in endothelia are mediated primarily via KDR [5]. The biological function(s) of Flt-1, which binds VEGF and is a specific receptor for the VEGF-related factors, placental growth factor (PIGF) and VEGF-B, have not yet been defined, and the signalling pathways mediating the actions of this receptor are not known. Current thinking favours the view that Flt-1 functions during embryogenesis primarily as a ‘decoy’ receptor that negatively regulates KDR-mediated actions of VEGF [1]. The primary

defect in Flt-1 null mice is an overproduction of endothelial progenitors [5]. Mice lacking only the Flt-1 kinase domain develop normally [6], indicating that Flt-1 intracellular signalling is not required for embryonic angiogenesis. Flt-1 can also directly modulate KDR-mediated biological responses [7]. It remains a matter for debate whether Flt-1 and specific ligands for this receptor can induce biological functions or signalling in endothelial cells [1]. However, Flt-1 mediates PIGF-induced migration and tissue factor expression in monocytes [8], and FAK tyrosine phosphorylation and chemotaxis in the monocyte/macrophage RAW 264.7 cell line [9]. Furthermore, PIGF and Flt-1 have been shown to mediate post-developmental pathophysiological neovascularisation in adult mice [10,11].

The Fos family of transcription factors play critical roles in cell proliferation and development [12,13], but their regulation by PIGF and VEGF in endothelial cells is poorly understood. The results presented in this paper show, for the first time, regulation of *FosB* mRNA by VEGF and PIGF, and indicate that in contrast to the effects of VEGF on expression of other endothelial genes, the ability of PIGF to induce *FosB* was similar to that displayed by VEGF. Furthermore, PIGF induced a similar response in Flt-1-expressing RAW 264.7 cells. These findings provide evidence that PIGF is able to generate intracellular signals leading to endothelial *FosB* expression as effectively as VEGF, and indicate that *FosB* may play a role in mediating biological actions of PIGF via Flt-1.

2. Materials and methods

2.1. Cell culture and VEGF treatment

Human umbilical vein endothelial cells (HUVEC) were obtained from TCS CellWorks Ltd and grown in EBM medium (Clonetics) supplemented with 10% foetal bovine serum (FBS), 10 ng/ml human epidermal growth factor, 12 µg/ml bovine brain extract and 50 µg/ml gentamicin sulphate. Wild type (w.t.) and Flt-1-transfected porcine aortic endothelial (PAE) cells (kind gift of Dr L. Claesson-Welsh, Department of Genetics and Pathology, Uppsala University, Sweden) were grown in Ham's F-12 medium (Invitrogen) supplemented with 10% FBS and either 100 U/ml penicillin and 100 µg/ml streptomycin (PAE/w.t.) or 500 µg/ml G418 sulphate (PAE/Flt-1). Murine RAW 264.7 cells (European Collection of Cell Cultures) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Recombinant human (h) and mouse (m) growth factors (hVEGF₁₆₅, hPIGF-1, mVEGF₁₆₄, mVEGF₁₂₀ and mPIGF-2) were obtained from R&D Systems and used at 25 ng/ml (0.6–0.9 nM). For growth factor stimulation experiments, confluent cultures of HUVEC and PAE cells were rendered quiescent by incubation in supplement-free, reduced serum (0.3%) medium overnight. RAW 264.7 cells were used at 70% confluence. Prior to addition of growth factors, cells were washed once with serum and supplement-free medium and then incubated in serum and supplement-free medium with or without the addition of growth

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factors for 20 min to 24 h. At the end of incubations, cells were washed twice with ice-cold phosphate-buffered saline and RNA subsequently extracted.

2.2. RNA isolation and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. During RNA purification an on-column DNase digestion was performed using the RNase-free DNase set (Qiagen) to remove any residual chromosomal DNA. First strand cDNA was synthesised from 2 µg of each DNA-free total RNA sample with Superscript II RNase H⁻ reverse transcriptase and oligo(dT)_{12–18} primer (Invitrogen). PCR reactions were performed on a PTC-100 programmable thermal controller (MJ Research) using the Taq PCR master mix kit (Qiagen) and gene-specific oligonucleotide primers (Table 1) synthesised by MWG Biotech Ltd. The PCR cycle number required for amplification to be within the exponential phase was experimentally determined for each primer pair. First strand cDNAs were normalised with respect to expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR products were resolved on 1% agarose gels and visualised by ethidium bromide staining.

2.3. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed on the LightCycler system (Roche Diagnostics) using first strand cDNA (generated as above) in conjunction with the LightCycler-FastStart DNA Master SYBR Green I kit and gene-specific oligonucleotide primers (Table 1). Primers were designed using the public domain Primer 3 program (available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to amplify products between 200 and 300 bp in size, with an optimum of 250 bp. PCR reactions were set up and performed according to the manufacturer's instructions, with amplification specificity confirmed by melting point analysis. Data were normalised with respect to expression of GAPDH and scaled relative to expression in control unstimulated cells.

3. Results

The effects of hVEGF₁₆₅ and hPIGF-1 on expression of Fos genes in HUVEC were determined using real-time quantitative RT-PCR at different time points using gene-specific oligonucleotide primers and GAPDH as a reference gene. VEGF₁₆₅ and PIGF-1 both markedly induced expression of *FosB* with similar time courses. VEGF₁₆₅ and PIGF-1 induced detectable increases in *FosB* expression after 20 min, maximum effects of 43 ± 6 -fold and 37 ± 2 -fold above the control level after 45 min, respectively, and expression thereafter declined to basal levels after 3 h (Fig. 1A). *FosB* is expressed in two forms, a full-length form and an alternatively spliced variant, Δ *FosB*, encoding a truncated protein lacking part of the carboxy-terminal domain [14]. Semi-quantitative RT-PCR analysis of the *FosB* mRNAs induced by VEGF and PIGF showed upregulation of both mRNA species (Fig. 1B). VEGF and PIGF also both induced expression of *c-Fos*, but VEGF consistently produced a stronger induction of *c-Fos* compared with PIGF (Fig. 1C). Induction of *c-Fos* by both factors reached a maximum after 20 min and declined to the control level after only 90 min. Neither VEGF nor PIGF significantly increased expression of Fos-related antigen-1 (Fra-1), or members of the Jun family of transcription factors that heterodimerise with Fos, including c-Jun, JunB, and JunD (results not shown). Fra-2 expression was not detected in HUVEC (results not shown). Western blotting of whole cell or nuclear extracts prepared from HUVEC treated with either PIGF or VEGF did not detect c-Fos or FosB protein (results not shown).

Table 1
Primers used for semi-quantitative and real-time quantitative RT-PCR

Gene	Species	Primer Sequence / supplier	Annealing temperature	Product size (bp)
<i>FosB</i> ^(a)	human	sense 5'-TTCTGACTGTCCCTGCCAAT-3' antisense 5'-CGGGGTCAGATGCAAAATAC-3'	55°C	249
<i>FosB</i> ^(a)	mouse	sense 5'-TGCTCAGTCTTTCCCTCCTG-3' antisense 5'-CAATCCGACCACTCATTAC-3'	55°C	248
<i>FosB</i> / Δ <i>FosB</i> ^{(b)(c)}	human	sense 5'-GTCTGGAGTTTGTGCTGGTG-3' antisense 5'-CTCTCTCCCCATGTGTTTG-3'	52°C	432 (<i>FosB</i>) 292 (Δ <i>FosB</i>)
<i>c-Fos</i>	human	sense 5'-AGGAGAATCCGAAGGGAAG-3' antisense 5'-CAAGGGAAGCCACAGACATC-3'	52°C	247
<i>c-Fos</i>	pig	sense 5'-AGAATCCGAAGGGAAGGAA-3' antisense 5'-AGATCAAGGGAAGCCACAGA-3'	55°C	248
<i>TF</i>	human	sense 5'-CGACGAGATTGTGAAGGATG-3' antisense 5'-CGGAGGCTTAGGAAAGTGTG-3'	55°C	252
<i>GAPDH</i>	human	sense 5'-GTCAGTGGTGGACCTGACCT-3' antisense 5'-CCCTGTTGCTGTAGCCAAAT-3'	55°C	251
<i>GAPDH</i>	pig	sense 5'-CAGTCAAGGCGGAGAACG-3' antisense 5'-GCAGAAGGGGCGAGATG-3'	55°C	202
<i>GAPDH</i>	mouse	sense 5'-AAAATGGTGAAGGTCGGTGT-3' antisense 5'-GTTAGTGGGGTCTCGCTCCT-3'	55°C	249
<i>GAPDH</i> ^(c)	human / mouse	sense 5'-ACCACAGTCCATGCCATCAC-3' antisense 5'-TCCACCACCCTGTTGCTGTA-3'	60°C	452
<i>KDR</i> ^{(c)(d)}	human / mouse	R&D Systems, VEGF R2 Primer Pair	55°C	569
<i>Flt-1</i> ^{(c)(d)}	human / mouse	R&D Systems, VEGF R1 Primer Pair	55°C	305 (human) 302 (mouse)

^(a) Primers do not distinguish between *FosB* and Δ *FosB*, yielding an identical product with both.

^(b) Primers flank the 140 bp intron retained in *FosB* and spliced out in Δ *FosB*.

^(c) Primers used for semi-quantitative RT-PCR.

^(d) Primer sequences not provided by supplier.

It was investigated whether PlGF had any effect on other known VEGF-induced genes. In particular, since PlGF has previously been reported to increase surface expression of tissue factor in monocytes [8], it was examined whether PlGF could induce tissue factor mRNA. As shown in Fig. 1D, VEGF produced a striking induction of tissue factor mRNA that was rapid, reaching a maximum increase above the control after 45 min, and thereafter declined slowly to near the control level after 24 h. In contrast, PlGF caused

no detectable increase in tissue factor mRNA expression over the same time course. In addition, PlGF also had no significant effect on several other genes that were strongly upregulated by VEGF, including *Egr3* and the nuclear receptor Nur77 [15].

The role of Flt-1 in mediating induction of *FosB* and *c-Fos* mRNAs by VEGF and PlGF was further investigated in cells expressing Flt-1 with no endogenous KDR/Flk-1 expression. In PAE cells stably transfected with Flt-1 (PAE/Flt-1), PlGF

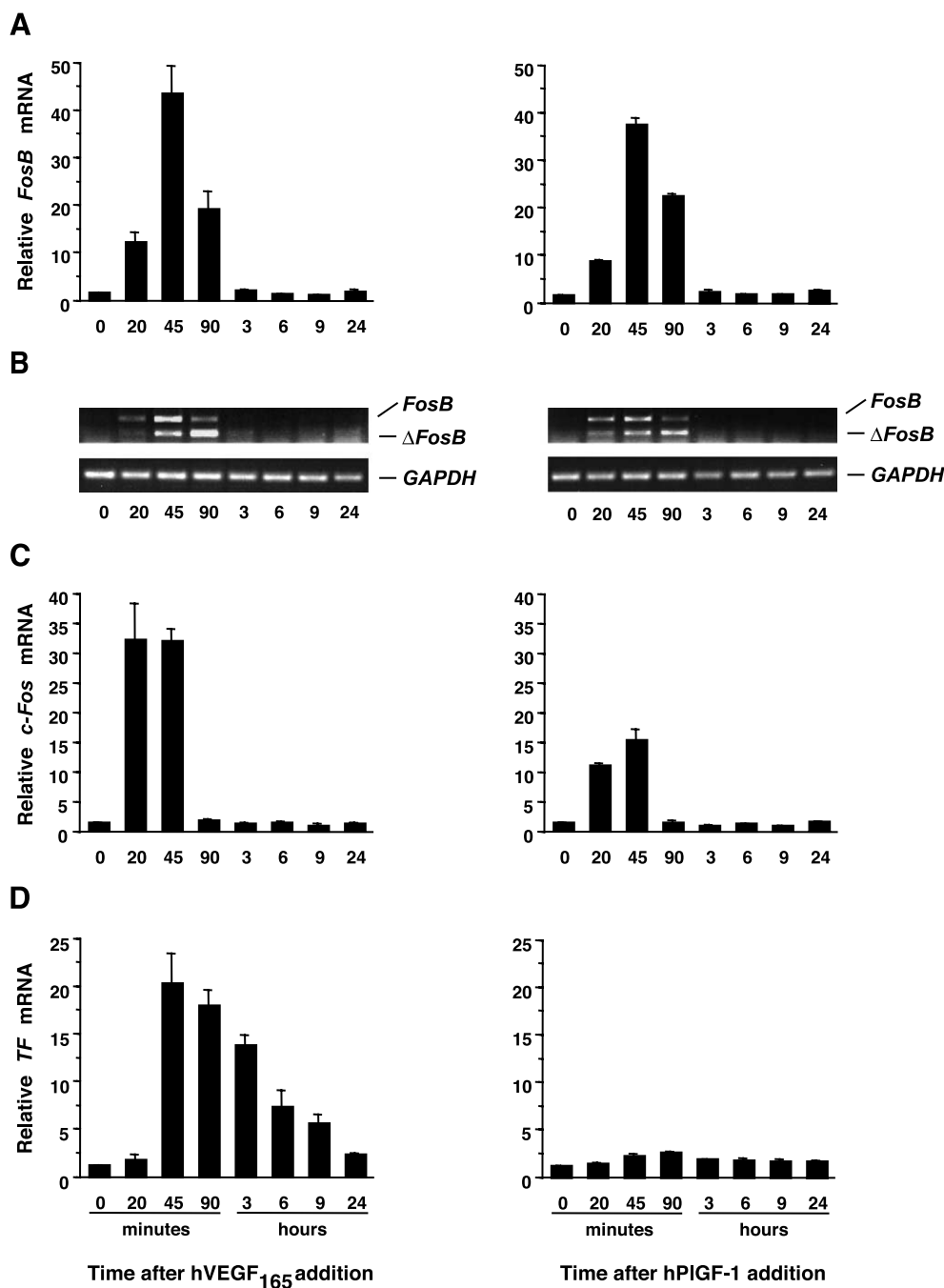


Fig. 1. Effects of VEGF₁₆₅ and PlGF-1 on *FosB* and *c-Fos* mRNA expression in HUVEC. Confluent cultures of HUVEC were serum-starved overnight, and treated for the times indicated with 0.6 nM (25 ng/ml) of hVEGF₁₆₅ (left panels) or 0.9 nM (25 ng/ml) hPlGF-1 (right). Expression of *FosB* (A,B), *c-Fos* (C) and tissue factor (*TF*, D) mRNA levels were determined by real-time quantitative RT-PCR (A,C,D) and normalised to levels of the housekeeping gene *GAPDH* or by semi-quantitative RT-PCR (B). The results in A, C and D represent the means \pm S.D. of three experiments. In B, the products of *FosB* RT-PCR were resolved into species corresponding to full-length (*FosB*) and truncated (Δ *FosB*) mRNAs. *GAPDH* mRNA levels are shown as a control reference gene (lower panel). The results shown are representative of four experiments.

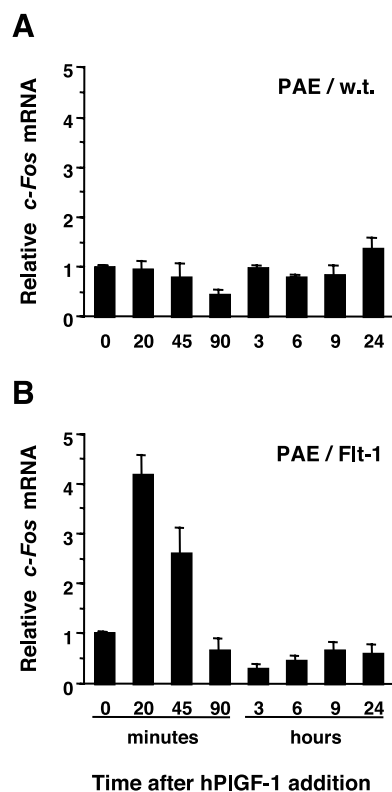


Fig. 2. Regulation of *c-Fos* expression in PAE/Flt-1 cells. Confluent and quiescent PAE/w.t. (A) and PAE/Flt-1 (B) cells were treated with 0.9 nM (25 ng/ml) hPIGF-1 for the times indicated. Porcine *c-Fos* mRNA levels were determined by real-time quantitative RT-PCR and normalised to *GAPDH* mRNA levels. The results shown represent the mean of three experiments \pm S.D.

stimulated *c-Fos* expression, but had no effect in the parental wild-type PAE cells (Fig. 2). VEGF had a similar effect on *c-Fos* expression with maximum fold increases at 20 min of 3.9 ± 0.5 and 3.6 ± 0.1 in PAE/KDR cells and PAE/Flt-1 cells, respectively (results not shown). The lack of an available sequence for the porcine *FosB* gene prevented analysis of *FosB* expression in PAE/Flt-1 cells.

Regulation of *Fos* genes through a PIGF/Flt-1 pathway was

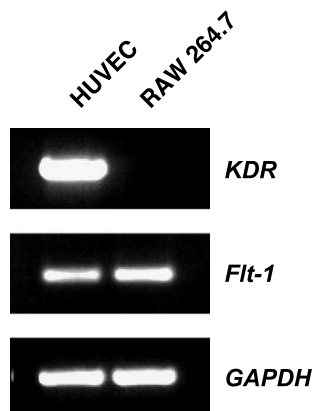


Fig. 3. *KDR* and *Flt-1* expression in HUVEC and RAW 264.7 cells. *KDR* and *Flt-1* mRNA levels were determined by semi-quantitative RT-PCR, using human/mouse gene-specific PCR primers. Corresponding *GAPDH* mRNA levels are shown. The results shown are representative of four experiments.

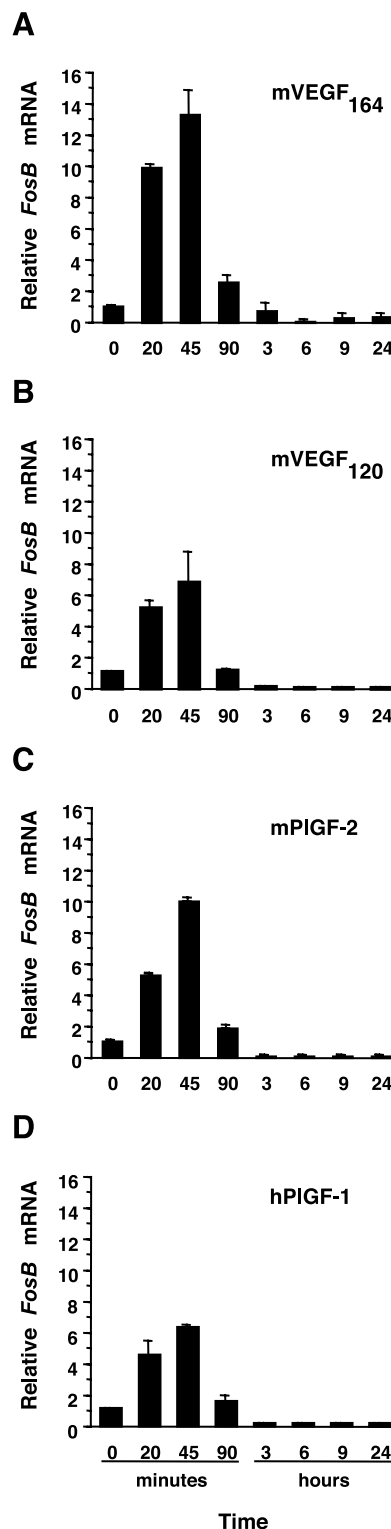


Fig. 4. PIGF induces *FosB* mRNA expression in RAW 264.7 cells. RAW 264.7 cells were treated for the times indicated with 0.6 nM mVEGF₁₆₄ (A), 0.9 nM mVEGF₁₂₀ (B), 0.6 nM mPIGF-2 (C), or 0.9 nM hPIGF-1 (D). All growth factors were used at 25 ng/ml. *FosB* mRNA levels were determined by real-time quantitative RT-PCR and normalised to *GAPDH* mRNA levels. The results shown represent the means \pm S.D. of three experiments.

next examined in the mouse monocyte/macrophage RAW 264.7 cell line, which has previously been shown to express Flt-1, but not KDR/Flk-1, and to display Flt1-mediated responses [9]. Evaluation of VEGF receptor mRNA expression in RAW 264.7 cells showed that these cells expressed Flt-1, but lacked detectable KDR expression (Fig. 3). Treatment of RAW 264.7 cells with the Flt-1 ligands, mVEGF₁₆₄, mVEGF₁₂₀, mPIGF-2, or hPIGF-1, induced *FosB* mRNA (Fig. 4). All these Flt-1 ligands also induced *c-Fos* expression (results not shown).

4. Discussion

These findings demonstrate for the first time that VEGF and the Flt1-specific ligand PIGF are able to induce mRNA expression of the transcription factor FosB and indicate that these factors also upregulate *c-Fos* expression though with different efficacies. The fact that PIGF and VEGF elicited a similar fold increase in *FosB* expression with similar kinetics, in both HUVEC and Flt-1-expressing RAW 264.7 cells, strongly indicates that Flt-1 is able to mediate induction of this gene as effectively as KDR. The stronger induction of *c-Fos* by VEGF compared with the effect of PIGF suggests that Flt-1 mediates a weaker *c-Fos* induction than KDR. The fact that PIGF was not able to induce significant expression of several other immediate early genes strongly upregulated by VEGF, including tissue factor, suggests that FosB may be one of a distinct subset of genes commonly regulated by VEGF and PIGF via Flt-1.

Though the members of the Fos family (*c-Fos*, FosB, Fra-1 and Fra-2) are among the best studied immediate early genes, their roles in VEGF signalling and functions are poorly understood and have been the subject of little investigation. Fos proteins only heterodimerise with members of the Jun family to form a large array of AP-1 transcription factor complexes, which are implicated in a variety of different cellular and biological processes [12]. The major phenotype of mice deficient in *c-Fos* is a lack of osteoclasts resulting in osteopetrosis, whereas ectopic transgenic expression results in osteoblast transformation leading to osteosarcoma [13]. Mice lacking *FosB* are viable, but display a profound deficiency in nurturing of young animals resulting in greatly increased post-natal mortality in their offspring [16]. Δ FosB is a naturally occurring truncated form of FosB arising from alternative processing of the *FosB* mRNA, to yield an mRNA species encoding a protein lacking the transactivation domain and TBP (TATA binding protein) binding motif [14]. Transgenic overexpression of Δ FosB in mice inhibits adipogenesis, and independently increases bone formation leading to osteosclerosis [17], while overexpression of Δ FosB specifically in thymocytes causes aberrant T cell development [18]. VEGF and PIGF similarly induced expression of both the full-length *FosB* and Δ FosB transcripts in HUVEC. Since Δ FosB can act in a dominant negative fashion to regulate transcription mediated via Fos, FosB and Jun proteins, upregulation of this form may act to regulate activity of full-length FosB downstream of Flt-1.

Despite the critical role of Flt-1 in angiogenesis revealed by Flt-1 null mice, it has proved extremely difficult to establish unequivocally that VEGFR1 or specific ligands for this receptor can mediate biological effects or signal transduction in endothelial cells. Earlier studies largely failed to find evidence

for PIGF stimulation of endothelial cellular functions such as migration or proliferation in cells naturally expressing Flt-1, or in PAE/Flt-1 cells. Flt1 is a weaker tyrosine kinase than KDR/Flk-1 [19,20], and though Flt-1 has been shown to mediate tyrosine phosphorylation of phospholipase C γ and activation of extracellular signal-regulated kinase [21,22], several studies have yielded varying and sometimes conflicting results regarding activation of signalling pathways in Flt-1-expressing cells, and PIGF has generally been found to be weaker than VEGF in stimulating signalling pathways [23–26]. The findings presented here provide one of the first indications that PIGF acting via Flt-1 can induce gene expression as effectively as VEGF, and identify FosB and *c-Fos* transcription factors as candidate mediators of Flt-1-mediated cellular functions.

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